RayBio®Biotin Label-based Antibody Array Trail Kit# 1

For the Simultaneous Detection of the Expression Levels of Multiple Target Proteins in Cell Culture Supernates, Serum and Plasma.

User Manual
(Glass slide-based Array)

RayBio® Biotin Label-based Antibody Array Human Trail Kit#-1 (Cat#: AAH-BLG-TK1): detect human target proteins

RayBio® Biotin Label-based Antibody Array Mouse Trail Kit#-1 (Cat#: AAM-BLG-TK2): detect mouse target proteins

RayBio® Biotin Label-based Antibody Array Rat Trail Kit#-1 (Cat#: AAR-BLG-TK3): detect rat target proteins

RayBiotech, Inc.

As the Protein Array Pioneer Company, Excellence and Innovation Is Our Goal

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RayBiotech, Inc

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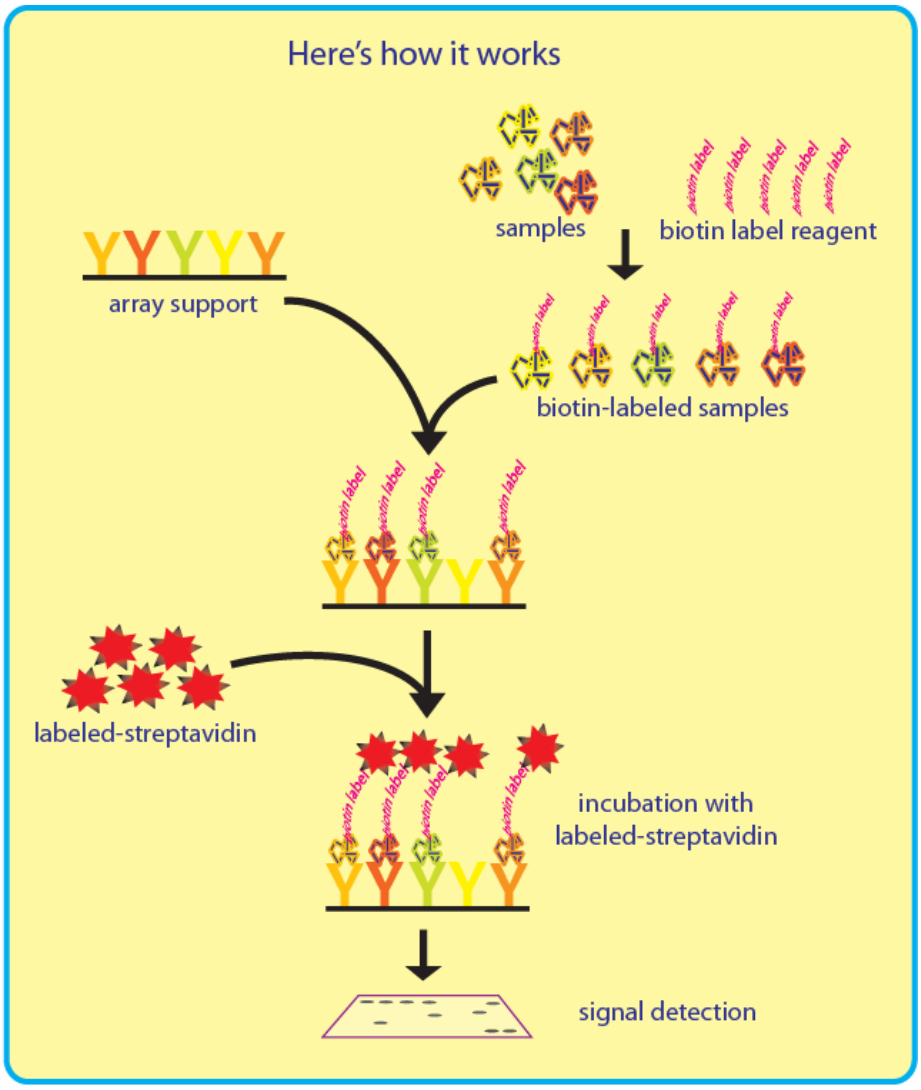
I. Introduction

Recent technological advances by Raybiotech have enabled the largest commercially available antibody arrays to date. With our label-based antibody arrays, researchers can now obtain a broad, panoramic view of cytokine expression. The expression levels of several hundreds of target proteins can be simultaneously detected, including cytokines, chemokines, growth factors, angiogenic factors, proteases, soluble receptors, and other proteins in cell culture supernate and other body fluids. Furthermore, an internal control is used to monitor the whole process including biotin-labeling, so this massive array will accurately reflect the available cytokines in your sample.

The first step in using the RayBio® Biotin Label-based Antibody Array is to biotinylate the primary amine of the proteins in cell culture supernates, serum and plasma. The biotin-labeled sample is then added onto glass chip and incubated at room temperature. Fluorescent dye-Conjugated Streptavidin (cy3 equivalent) is used to visualize the signals.



II. Materials Provided

Upon receipt, the kit should be stored at -20 °C. Please use within 6 months from the date of shipment. After initial use, the Blocking Buffer, Stop Solution, 20X Wash Buffer I and II, Serum Buffer and Fluorescent dye-Conjugated Streptavidin (cy3

equivalent) should be stored at 4 °C to avoid repeated freeze-thaw cycles. The Array 1 Glass Chip and internal control should be kept at -20 °C.

- Dialysis tube (Item A, 8 tubes)
- Labeling Reagent (Item B, 2 tubes)
- Internal control (Item C, 2 tubes)
- Stop Solution (Item D, 1 tube)
- RayBio® Biotin label-based antibody array trail kit# 1 or trail kit# 2 or trail kit# 3 Glass Chip with Frame (Item E, each slide with 4 Subarrays)
- Blocking Buffer (Item F, 8 ml)
- 20X Wash Buffer I (Item G, 30ml)
- 20X Wash Buffer II (Item H, 30ml)
- Fluorescent dye-Conjugated Streptavidin (Item I, cy3 equivalent, 2 tubes)
- Adhesive film (Item J)
- Serum Buffer (Item K, 8 ml)
- D-Tube Floating Rack
- 30 ml centrifuge tube

III. Additional Materials Required

- 1X PBS, pH=8.0
- Orbital shaker
- 1 ml tube
- 15 ml and 50 ml conical collection tube
- Laser scanner for fluorescence detection
- Aluminum foil

- Distilled water

IV. Overview and General Considerations

A. Handling glass chips

- The microarray slides are sensitive, so do not touch the array surface by tips, forceps or hand. Hold the slides by the edges only.
- Handle the slides with latex free gloves.
- Avoid breaking the glass slide.
- A clean environment is essential
- Remove the final buffer by gently applying suction with a pipette to corners of each chamber (see the following picture). Do not touch the array, only the sides.

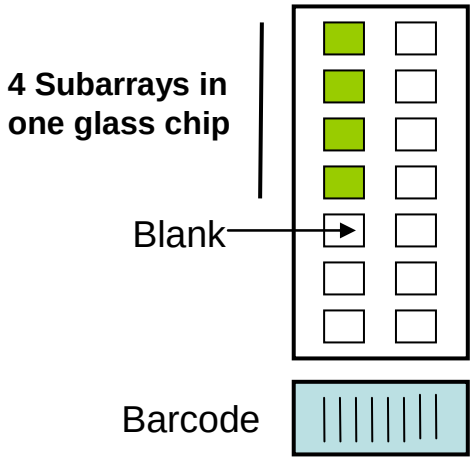
B. Incubation of Antibody Array

- Completely cover the array area with sample or buffer during incubation, and cover the incubation chamber with adhesive film or a plastic sheet protector to avoid drying, particularly when incubation is more than 2 hours.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.

- Avoid cross-contamination by preventing overflow to neighboring wells.
- Several incubation steps such as step 2 (sample incubation) in page 10, or step 7 (Fluorescent dye-Conjugated Streptavidin incubation) in page 11 may be done at 4 °C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.
- Avoid array slide exposure to light since step 6 in page 11.

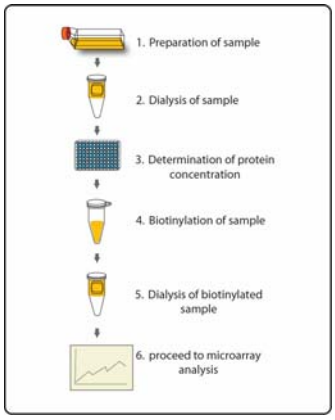
V. Protocol

Layout of Array Glass Chip

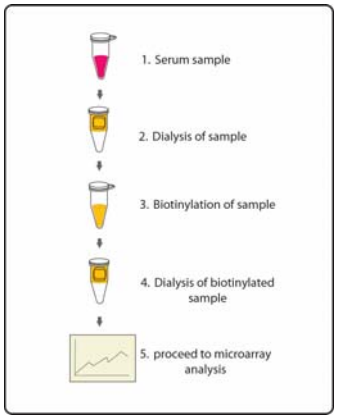


Assay Diagram

1. Cell Culture Supernates


1. Preparation of sample
2. Dialysis of sample
3. Determination of protein concentration
4. Biotinylation of sample
5. Dialysis of biotinylated sample
6. proceed to microarray analysis

2. Serum or Plasma


1. Serum sample
2. Dialysis of sample
3. Biotinylation of sample
4. Dialysis of biotinylated sample
5. proceed to microarray analysis

A. Preparation of Cell Culture Supernates

The cell culture supernates can be prepared in the following conventional manner:

To prepare cell culture supernates (cell conditioned media), cells are plated in 100 mm tissue culture dishes at a density of $1x10^6$ cells (*) per dish. The cells are then cultured with complete culture medium for 24~48 hours (**). The complete culture medium is replaced with lower serum medium such as 0.2% FCS serum, and then the cells are cultured for 48 hour (**) again once more. The supernates are collected, centrifuged at 1,000 g for 10 min, aliquoted and stored at –80 ^{0}C until use. Meanwhile, the cells are also collected and the total protein concentration is determined. For each sample it is recommended that the concentrations of the supernates and cell lysates (help nomalize different cell culture supernates) are determined using a total protein assay (BCA Protein Assay Kit, Pierce, Prod# 23227).

*Note: * The density of cells per dish used is dependent on the cell type More or less cells may be used.*

*** The culture time may be different and depends on your cell lines and research.*

B. Dialysis of Sample

The cell culture supernates, serum or plasma should be dialyzed with a Dialysis tube (Item A) before the biotin-labeling procedure. We recommend loading 200 μl cell culture supernates or 5-fold diluted serum/plasma with 1X PBS (pH=8) (20 μl serum or

plasma + 80 µl 1X PBS) into a dialyzer and dialyzing with at least 500 ml 1X PBS buffer (pH= 8) at 4 ^{0}C. Change the 1X PBS buffer and dialyze again. Allow at least 3 h for each dialysis step, stir gently.

Note: Preparation of 1X PBS, pH=8.0, 0.6 g KCl, 24 g NaCl, 0.6 g KH_2PO_4, 3.45 g Na2HPO4 dissolve in 2500 ml deionized or distilled water. Adjust pH=8.0 with 1M NaOH and adjust final volume to 3000 ml with deionized or distilled water.

C. Biotin-labeling Sample

Avoid contamination with any solution containing amines (i.e., Tris, glycine) as well as Azide during the biotinylation process.

1. Briefly spin down Internal Control tube (Item C*)* before use. Add 100 µl 1X PBS, pH=8.0 into the Internal Control tube, pipette up and down to dissolve the powder.

a) For labeling cell culture supernates: add 40 µl prepared Internal Control into each tube containing 180 µl dialyzed cell culture supernates.

b) For labeling serum or plasma: add 40 µl prepared Internal Control into each tube containing 35 µl dialyzed serum or plasma sample and 155 µl Serum Buffer (Item K).

2. Immediately before use, briefly spin down the Labeling Reagent tube (Item B). Add 100 µl 1X PBS into the

tube, pipette up and down or vortex slightly to dissolve the powder to prepare 1X Labeling Reagent Solution.

3. Add an appropriate amount* of prepared Labeling Reagent into above tube with sample in step 2, mix well immediately. Incubate the reaction solution at room temperature with gentle shaking for 30 min. Gently tap the tube to mix the reaction solution every 5 min.

* *For labeling cell culture supernates: 36 µl of 1X Labeling Reagent Soluition for labeling 1 mg total protein in the cell culture supernates.*
For labeling serum or plasma: Add 22 µl of Labeling Reagent Solution into each prepared serum or plasma sample in page 8 step 1.

4. Add 5 µl Stop Solution (Item D) into above reaction solution and immediately dialyze as directed in Step B.

5. Samples should be centrifuged at 10,000 g for 5 min (4°C) after Dialysis.

Note: Samples (supernates) can be stored at -20 or -80 °C until microarray assay.

D. Dry the Glass Chip

Open the box containing glass chip, and take it out, and then let it air dry for 1 hour in a clean environment before use.

Note: Protect the chip from dust or others contaminants.

E. Blocking and Incubation of Antibody Array

1. Add 100 µl of Blocking Buffer (Item F) into each well (Glass Chip with Frame, Item E) and incubate at room temperature for 30 min to block slides. Make sure there are no bubbles in the well.

2. Decant Blocking Buffer from each well (make sure to remove all of buffer). Add 100 µl of each sample into appropriate wells. Incubate arrays with sample at room temperature with gentle shaking for 2 hours or 4 °C for overnight.

   Note: a. We recommended dilution of the biotin-labeled cell culture supernates 2-10 fold or the biotin-labeled serum/plasma 40-fold with blocking buffer. Make sure there are no bubbles in the wells.

   b. The dilution used depends on the abundance of target proteins. The sample can be diluted further if the background or signal is too strong. If the signal is too weak, more sample can be used.

   c. *Incubation may be done at 4 °C overnight.*

   d. *Avoid the flow of samples into neighboring wells.*

3. Decant the samples from each well, and wash 3 times with 200 µl of 1X Wash Buffer I (Item G) at room temperature with gentle shaking for 5 min per wash.

4. Put the glass chip with frame into a box with Wash Buffer I

(cover the whole glass slide and frame with Wash Buffer I), and wash at room temperature with gentle shaking for 20 min.

5. Decant the Wash Buffer I from each well, Put the glass chip with frame into the box with Wash Buffer II (cover the whole glass slide and frame with Wash Buffer II), and wash 2 times at room temperature with gentle shaking for 5 min.

6. Remove all of Wash Buffer II in the well. Add 100 µl of 1X Fluorescent dye-Conjugated Streptavidin to each subarray. Cover the incubation chamber with Adhesive film, and then cover the plate with aluminum foil to avoid exposure to light or incubate in dark room.

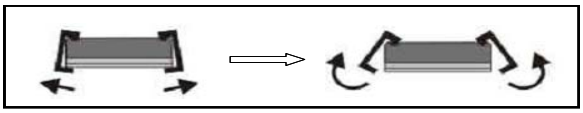
   Note: briefly spin down the Fluorescent dye-Conjugated Streptavidin (Item I) before use. Add 50 µl of Blocking Buffer into the tube to prepare a Streptavidin Concentrate. Pipette up and down to mix gently (don't store the Concentrate for next day use). Add 5 µl of Streptavidin Concentrate into a tube with 500 µl of Blocking Buffer. Mix gently to prepare 1X Streptavidin solution.

7. Incubate at room temperature with gentle shaking for 2 hours.

   Note: Incubation may be done at 4 °C for overnight.

8. Decant the solution and disassemble the slide out of the incubation frame and chamber. Disassemble the device by pushing clips outward from the slide. Carefully remove the slide from the gasket.

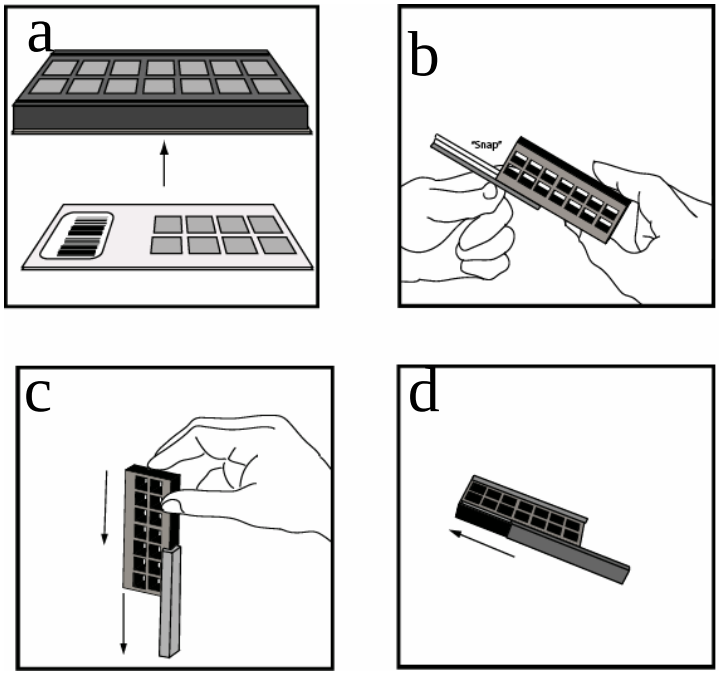
Note: Be careful not to touch the surface of the array side



9. Gently put the glass chip into a 50 ml centrifuge tube as shown in the following figure A or a plastic box with 40 ml of 1X Wash Buffer I. Gently roll the tube or shake for 10 min. Remove the wash buffer. Repeat 2 times for a total of 3 washes.

A)

B)



10. Wash the glass chip with 40 ml of 1X Wash Buffer II. Repeat one time for a total of two washes for 5 min per wash.

11. Finally wash the glass chip with 40 ml of deionized or distilled water for 5 min.

Note: You may assemble the glass chip into an incubation chamber by following step if necessary. You may want to practice assembling the device with a blank glass slide as shown in the figure B:

a. Apply slide to incubation chamber barcode facing upward as in step (a).
b. Gently snap one edge of a snap-on side as shown in step (b).
c. Gently press other of side against lab bench and push in direction shown in step (c)
d. Repeat with the other side.

F. Fluorescence Detection

Put the glass chip into a 50 ml centrifuge tube, and dry the glass chip by centrifuge at 1,000 rpm for 3 minutes, or, dry the glass chip by a compressed N_2 stream. You can also let the glass chip dry completely in a clean environment (protect from light). Make sure the slides are absolutely dry before the scanning procedure. The signals can be visualized with a laser scanner, such as the Axon GenePix, using the cy3 channel.

Note: We recommend scanning the slides right after the experiment. You also can store the slides at –20 °C in the dark for several days. If you do not have a laser scanner, we can provide this service for you. Just simply send your slide to us and we will take care of it.

VI. Interpretation of Results:

A biotinylated protein and internal control will produce positive control signals, which can be used to identify the orientation and help normalize the results from different arrays being compared. Antibody affinity varies significantly. The fluorescence intensity detected on the array with each antibody depends on its affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies.

The **RayBio® Analysis Tool** is a program specifically designed for analysis of RayBio® Label-based Antibody Arrays Trail Kit# 1. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste." Call RayBiotech, Inc. at 770-729-2992 for ordering information.

RayBio® Biotin Label-based Anitibody Array Trial Kit# 1 Map (Cat#: AAH-BLG-TK1)

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	POS 1	POS 2	POS 3	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
2	POS 1	POS 2	POS 3	NEG	NEG	EOTAXIN	EOTAXIN-2	GCSF	GM-CSF	ICAM-1	IFN-γ	I-309	IL-1α
3	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13
4	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-6sR	IL-7	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13
5	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ	RANTES	TGF-β1
6	IL-15	IL-16	IL-17	IP-10	MCP-1	MCP-2	M-CSF	MIG	MIP-1α	MIP-1β	MIP-1δ	RANTES	TGF-β1
7	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	NEG	NEG	IC 1	IC 2	IC 3	NEG	NEG
8	TNF-α	TNF-β	s TNF RI	s TNF RII	PDGF-BB	TIMP-2	NEG	NEG	IC 1	IC 2	IC 3	NEG	NEG

RayBio® Biotin Label-based Anitibody Array Trial Kit# 2 Map (Cat#: AAM-BLG-TK2)

	A	B	C	D	E	F	G	H	I	J
1	Pos 1	Pos 2	Pos 3	Neg	Neg	6Ckine	CTACK	Eotaxin	GCSF	GM-CSF
2	Pos 1	Pos 2	Pos 3	Neg	Neg	6Ckine	CTACK	Eotaxin	GCSF	GM-CSF
3	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
4	IL-2	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
5	IL-17	IFN-γ	KC	Leptin	MCP-1	MCP-5	MIP-1α	MIP-2	MIP-3 β	RANTES
6	IL-17	IFN-γ	KC	Leptin	MCP-1	MCP-5	MIP-1α	MIP-2	MIP-3 β	RANTES
7	SCF	sTNFRI	TARC	TIMP-1	TNF-α	Thrombopoietin	VEGF	IC 3	IC 2	IC 1
8	SCF	sTNFRI	TARC	TIMP-1	TNF-α	Thrombopoietin	VEGF	IC 3	IC 2	IC 1

RayBio® Biotin Label-based Anitibody Array Trial Kit# 3 Map (Cat#: AAR-BLG-TK3)

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS 1	POS 2	POS 3	NEG	Activin A	Agrin	B7-2/CD86	beta-NGF	CINC-1	CINC-2 alpha	CINC-3	CNTF
2	POS 1	POS 2	POS 3	NEG	Activin A	Agrin	B7-2/CD86	beta-NGF	CINC-1	CINC-2 alpha	CINC-3	CNTF
3	Fas Ligand	Fractalkine	GM-CSF	ICAM-1	IFN-gamma	IL-1 alpha	IL-1 beta	IL-1 R6	IL-2	IL-4	IL-6	IL-10
4	Fas Ligand	Fractalkine	GM-CSF	ICAM-1	IFN-gamma	IL-1 alpha	IL-1 beta	IL-1 R6	IL-2	IL-4	IL-6	IL-10
5	IL-13	Leptin	LIX	L-Selectin	MCP-1	MIP-3 alpha	MMP-8	PDGF-AA	Prolactin R	RAGE	Thymus Chemokine-1	TIMP-1
6	IL-13	Leptin	LIX	L-Selectin	MCP-1	MIP-3 alpha	MMP-8	PDGF-AA	Prolactin R	RAGE	Thymus Chemokine-1	TIMP-1
7	TNF-alpha	VEGF	NEG	NEG	NEG	NEG	NEG	NEG	NEG	IC 3	IC 2	IC 1
8	TNF-alpha	VEGF	NEG	NEG	NEG	NEG	NEG	NEG	NEG	IC 3	IC 2	IC 1

VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipettors and ensure correct preparation
	Short incubation times	Ensure sufficient incubation time and change sample incubation step to overnight
	Too low protein concentration in sample	Don't make too low dilution Or concentrate sample
	Improper storage of kit	Store kit at suggested temperature
High background	Sample is too concentrated	Use more diluted sample
	Excess of streptavidin	Make sure to use the correct amount of streptavidin
	Inadequate detection	Check laser power and PMT parameters
	Inadequate wash	Increase the volume of wash buffer and incubation time
Uneven signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completely covered by reagent	Completely cover arrays with solution

VIII. Reference List

1. Profiling of cytokine expression by biotin-labeled-based protein arrays. Ying Lin, Ruochun Huang, Lipai Chen *et al*. ***Proteomics.*** 2003, 3: 1750-1757.

2. Proteomic profiling of the cancer microenvironment by antibody arrays. Vladimir Knezevic, Chidchanok Leethanakul, Verena E. Bichsel *et al*. ***Proteomics*** 2001, *1*, 1271–1278

3. Antibody microarray profiling of Mouse prostate cancer sera: Antibody screening and identification of potential biomarkers Jeremy C. Miller, Heping Zhou, Joshua Kwekel, Robert Cavallo *et al*. The, Brian B. Haab. ***Proteomics.*** 2003, 3: 56-63.

4. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. Byung-Gyu Kim, Cuiling Li, Wenhui Qiao, Mizuko Mamura, Barbara Kasperczak, Miriam Anver, Lawrence Wolfraim, Suntaek Hong, Elizabeth Mushinski, Michael Potter, Seong-Jin Kim, Xin-Yuan Fu, Chuxia Deng and John J. Letterio. ***Nature.*** 2006; Vol 441: 1015

5. Connexin suppresses Mouse glioblastoma cell growth by down-regulation of monocyte chemotactic protein 1, as discovered using protein array technology. Ruochun Huang, Ying Lin, Cheng C. Wang, Jacob Gano, Biaoyang Lin, Qian Shi, Alton Boynton, Jocelyn Burke, and Ruo-Pan Huang. ***Cancer Res***. 2002;62:2806-2812.

6. LPS induces the interaction of a transcription factor, LPS-induced TNF-a factor, and STAT6(B) with effects on multiple cytokines. Xiaoren Tang, Deborah Levy Marciano, Susan E. Leeman and Salomon Amar. ***PNAS***. April 5, 2005 vol. 102 no. 14, 5132-5137

7. HIV-1-mediated apoptosis of neuronal cells: Proximal molecular mechanisms of HIV-1-induced encephalopathy. Yan Xu, Joseph Kulkoshy, Roger j. Pomerantz**.** ***PNAS***. 2004 May 4, 2004 Vol. 101 No. 18.

8. A novel method for high-throughput protein profiling from conditioned media and patient's sera. Ruo-Pan Huang**,** Ruochun Huang**,** Yan Fan, and Ying Lin. ***Ana. Biochem***. 2001;294(1):55-62.

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1. Antibody arrays
2. Cytokine antibody array
    - Human cytokine antibody arrays
    - Mouse cytokine antibody arrays
    - Rat cytokine antibody arrays
   - Pathway- or disease-focused antibody arrays
    - Inflammation antibody array
    - Angiogensis antibody array
    - Chemokine antibody array
    - Growth factor antibody array
    - MMP antibody array
    - Atherosclerosis antibody array
   - Antibody analysis tool, software

3. ELISA
4. Cell-based phosphorylation assay
5. Custom antibody arrays
6. Antibody
7. Recombinant protein
8. Cytokine protein arrays
9. Quantibody arrays for quantitative measurement of cytokine and other protein concentration.

10.Phosphorylation antibody arrays
11.Biotin label-based antibody arrays for high density antibody arrays

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3. Peptide synthesis
4. Production of recombinant protein and antibody
5. Peptide arrays
6. Phosphorylation arrays
7. ELISA

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